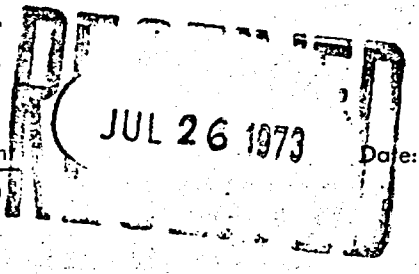
#924

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application for Research Grant
(Use extra pages as needed)

Date:

1. Principal Investigator (give title and degrees):

John Candee Houck, Ph.D. — Scientific Director, Research Foundation, Children's Hospital, Washington, D. C. — Professor of Child Health and Development, George Washington University Medical School

2. Institution & address:

Children's Hospital Research Foundation
2125 13th Street, N. W.
Washington, D. C. 20009

3. Department(s) where research will be done or collaboration provided:

Biochemical Research Laboratory

4. Short title of study:

Search for Lung Chalone

5. Proposed starting date: January 1 1974

6. Estimated time to complete: Three Years

7. Brief description of specific research aims:

(a) To isolate and establish in vitro pure cell lines of human squamous epithelial and "oat cell" carcinomas from resected lung tumors.

(b) To determine quantitatively the rate of proliferation of both of these cell lines in vitro from H^3 thymidine incorporation studies and by calculating their population doubling times.

(c) Using these two cell systems, the effects of lung tissue extracts upon their mitotic activity in vitro will be determined.

(d) Mitotic inhibitory activity for lung carcinoma cells in vitro from aqueous extracts of normal cadaver or animal lung will be characterized in terms of cell and tissue specificity, cytotoxicity, and chemistry (size, isoelectric point).

8. Brief statement of working hypothesis:

It has been proposed by Bullough and Laurence that most cells are under the control of a specific and endogenous negative feedback inhibitor of mitosis called "chalone". Chalones have been defined operationally for epidermis, fibroblast, lymphocyte, melanocyte, and a number of other cell types as indicated in the enclosed review reprint. Our hypothesis is that lung, too, is under the control of chalone, as has originally been suggested in a small paper by Simnett, Fisher, and Hepplesten, entitled "Tissue-specific inhibition of lung alveolar cell mitosis in organ culture" (Nature 223: 944, 1969). All chalones that have been described to date also specifically inhibit the mitotic activity of the appropriate tumor cell! Tumor cells require more chalone than do normal cells for an equal degree of inhibition. Chalone appears to inhibit both normal and tumor cells in the G_1 phase of the mitotic cell cycle. Therefor by adding lung chalone (if such exists) in vivo, all lung epithelial cell proliferation will cease; when the dose of chalone is reduced after 1 or 2 days, all the tumor cells will escape chalone control and enter in a synchronized fashion the "S" phase of the cell cycle, during which time they will be extremely vulnerable to chemotherapy by DNA poisons. The normal cells will still be inhibited by chalone in G_1 because they bind the chalone better than do the tumor cells and hence require 2-3 times less chalone for inhibition. By using lung chalone to specifically synchronize tumor cells in vivo, a profound increase in the efficacy of chemotherapy of pulmonary carcinoma might result. This possibility strongly directs us to search for and purify and characterize a chalone for lung.

9. Details of experimental design and procedures (append extra pages as necessary)

Specific Aim #1 is to establish in vitro cell lines of human pulmonary squamous epithelial carcinoma and "oat cell" carcinoma.

Both Georgetown University Hospital (Dr. John Potter) and the Washington Hospital Center are sister institutions of Children's Hospital in this city. At both these institutions, a number of lung resections are done every week. With the cooperation of the Departments of Pathology and Surgery at both of these institutions, we will be able to obtain tissue samples from lung resections which have been diagnosed pathologically to be pulmonary squamous epithelial carcinoma or "oat cell" carcinoma. The tumor samples will be introduced into three media: (1) Medium 199 (a complete general medium) containing 20% serum, (2) L-15 containing 20% serum which is a medium uniquely poor in glucose, highly rich in amino acids, and particularly good for tumor cells, and (3) McCoy's Medium containing 20% serum, which has 4 X as much glucose as does Medium 199 and is also helpful for growing certain kinds of tumor cells. All these media will be supplemented as needed with Pen-Strep and Fungizone to limit the bacterial and fungal contaminant growth in vitro of these explants. After 10 days to 2 weeks, a large number of cells will start to proliferate from the explant onto the glass wall of the incubation vessels. At this point, the serum concentration in the medium will be reduced to 10% and 1 mg/ml of fibroblast chalone (1) will be introduced into the vessels. Preliminary evidence indicates that the mitotic activity of the fibroblasts from lung tissue explant will be reduced by over 90% at this concentration of chalone (1). In this fashion, we would hope that within a few weeks we will have cultivated pure lung carcinoma cells, free of both microbial, fungal, and fibroblast contamination.

These cells, which adhere to glass coverslips in Leighton tubes, will be harvested at various periods of time after seeding and their histology will be determined both at the light microscope and electron microscopic levels.

Specific Aim #2 is to determine proliferative rates in vitro.

The proliferative rate of these two human lung tumor cell types in vitro will be determined by two different techniques:

(a) By determining the rate of incorporation of tritiated thymidine into macromolecular DNA in a manner analogous to that described by us for fibroblasts in vitro (1). Thus cultures at various days after seeding a known number of cells (determined in a hemocytometer) into replicate Leighton tubes will be incubated for various periods of time (ranging from 1 to 24 hrs) with a single pulse of 1 µci of H^3 thymidine. At the conclusion of the incubation,period, these cells will be rinsed with ice cold isotonic saline to remove free isotope, serum, and medium, and then precipitated in the Leighton tube with 5% trichloracetic acid. A rubber policeman will be used to collect all this precipitate,

which will be washed twice with TCA and then solubilized in the usual fashion using an NBZ solubilizer. The total uptake of H^3 thymidine into macromolecular DNA will be determined by liquid scintillation counting in the usual fashion. From these data, the rate of thymidine incorporation by these cells during cultivation may be quantitatively compared with their cell number and the age of the culture.

(b) Proliferative rates of these cells may also be determined, since they adhere to glass, in the manner developed by Raff and Houck (2). A known number of cells are seeded into replicate Leighton tubes and at various periods of time after this inoculation of cultured cells, the numbers of cells are counted, using a Whipple eyepiece and an inverted microscope. In this fashion, non-destructive analysis of the number of cells in each culture may be obtained daily or twice daily for a week until the cells are so dense as to defy differentiation by the Whipple eyepiece. A plot of the log of the number of cells in these cultures versus time will give an initial straight line, the slope of which will permit the calculation of the time required for the population of cells to double. This population doubling time represents a quantitative measure of the proliferative rate of cells cultivated in vitro (2).

In this fashion then, we will have two measures of the proliferative rate of human lung carcinoma cells in culture in order to explore the possibility that the lung cells are under chalone control.

Specific Aim #3 is to demonstrate the mitotic inhibition by pulmonary tissue extracts.

Using the in vitro-cultivated lung tumor cells described above and measuring the two parameters of proliferation indicated above, extracts of animal and cadaver pulmonary tissue (from the Department of Pathology at Children's Hospital) will be obtained via the extraction of macerated lung tissue into 10 ml of 0.15M NaCl in the cold, using an Omni Mixer as has been described by us for other tissues (3, 4). This extract will be allowed t o stand overnight in the cold in order to swell and thereby permit molecular diffusion. Subsequently, the tissue brei will be centrifuged in the cold and the clear supernatant removed and subjected to Amicon Daiflo ultrafiltration, using a membrane which will pass molecules of 500 daltons or less. In this fashion, large volumes of pure water may be used to "wash" the extract, removing thereby both salt and small molecular weight materials such as cold thymidine which might, by diluting the pool size of the H^3 labeled thymidine, complicate the measurements of the proliferative rates of the cells when using the thymidine uptake technique. After seven volumes of clear water have been washed through the extract in the Amicon Diaflo filtration system, the extract will be concentrated by using nitrogen pressure to express a large amount of water through the filter from the extract. This concentrated material will then be lyophilized.

Various concentrations of lyophilized material will be made up in the appropriate medium and added to the cultures of lung tumor-derived cell lines mentioned above. The effects of these extracts upon the cell proliferation rate of both cell types will be determined quantitatively. The results of this experiment will dictate the subsequent steps: (a) if there is no apparent inhibition by this technique and careful repetition confirms this, at this point the project is over; (b) if on the other hand, there is evidence of inhibition of mitosis from both techniques, then further characterization and purification of the inhibitory principle in this tissue extract seems mandatory.

Specific Aim #4 is to characterize the mitotic inhibitory activity of pulmonary tissue extracts.

A number of steps will be involved in the characterization of the apparent mitotic inhibition, namely (a) biological, and (b) chemical. The biological studies will be addressed to the question of the specificity of this apparent mitotic inhibition of pulmonary carcinoma cells in culture. Specifically, various concentrations of lung extract described above which have been shown to inhibit the proliferative rate of these pulmonary tumor cells in vitro will be tested upon other cell types available in this

laboratory: diploid human fibroblasts, human lymphocytes which have been stimulated to transform via either lectins or antigens, HeLa cells, colon carcinoma cells, and human choriocarcinoma cells. If the specificity of the chalone concept is borne out for lung tissue, this lung extract, while it might be contaminated with fibroblast chalone, should in fact have no effect on human colon, chorion, lymphocyte, or HeLa cells in vitro. Extracts of tissues other than lung, such as muscle, kidney, liver, will be tested at the same concentration as the lung extract upon the cultured cells of pulmonary carcinoma as described above in an attempt to demonstrate that only extracts of lung tissue have material in them capable of inhibiting the proliferation of these cells.

Finally, the question of cytotoxicity is enormously important in the search for chalone, namely, according to Houck's Law(5), "dead cells don't divide," and the corollary of this is that "dying cells divide damn slowly" (see enclosed review reprints). Our primary measures for studying the viability of cells cultivated in vitro is firstly by determining their ability to exclude a vital dye, secondly by the reversibility of the mitotic inhibition subsequent to the removal of the extract being tested from the culture, and finally by determining whether any inhibition of initial rates of incorporation into the cells of C^{14} labeled amino acids into macromolecular protein has occurred. Cytotoxic agents usually are found to inhibit this protein synthetic rate very quickly. However, with specific DNA synthesis inhibition, usually after some time there is a marked inhibition of the rate of protein synthesis by the inhibited cell. Therefore only the effects of chalone upon the initial rates of protein synthesis will be terribly helpful in making a judgment to include or exclude cytotoxicity as a mechanism of the mitotic inhibition of lung tissue extracts upon these cells.

The initial chemical characterization of this mitotic inhibitory activity will be through sequential fractionation on the basis of molecular size, using Amicon Diaflo filters in series (6). In this fashion, we can accumulate large amounts of the crude extracts on the basis of molecular weights into weight ranges above 300,000 daltons; between 300,000 and 100,000 daltons; between 100,000 and 50,000 daltons; between 50,000 and 30,000 daltons; between 30,000 and 10,000 daltons; and finally between 10,000 and 1,000 daltons. This type of molecular sieving has been done by us for fibroblast mitogen (7), fibroblast chalone (1), and lymphocyte chalone (8, 9). All of these molecular weight ranges can be tested for their cytotoxic-free inhibition of the proliferation rate of pulmonary carcinoma cells in vitro as described above. Hopefully only one of these molecular weight ranges will contain cytotoxic-free mitotic inhibitory activity. This molecular weight range material will then be subjected to isoelectric focusing (10) after determining that the mitotic activity is stable upon standing in high concentrations of sucrose for 2-3 days at various pH (7). Isoelectric focusing will firstly demonstrate the isoelectric point of the material and secondly provide an enormously large purification of this material.

Specific Aim #5 is to isolate, purify, and chemically characterize this material.

Using in sequence molecular sieving by ultrafiltration and isoelectric focusing, the subsequent partially purified inhibitor of the proliferation of pulmonary carcinoma cells in vitro will be subjected to preparative acrylamide gel electrophoresis (11) and the active electrophoretic fraction will be determined as described above by studies of the inhibition of the proliferative rates of these pulmonary carcinoma cells. The active material from this step will be subjected to analytical acrylamide gel electrophoresis at three different pH and in the presence of sodium dodecyl sulfate (7). If the material is not homogeneous, at this point in the purification scheme as judged by these rigorous criteria, then further purification steps will be employed, namely column chromatography, using DEAE cellulose at slightly alkaline pH and a salt gradient, or CM cellulose at pH 4.5 using a salt gradient. Again, the active fraction will be determined biologically as described above, and its homogeneity will be determined by acrylamide gel electrophoresis

at three different pH and SDS electrophoresis. At some point during this process, essential homogeneous material should result. At this time, the molecular weight of this material will be determined firstly by its elution from columns of P200 or P100 or P50 or P10, depending on molecular weight (12), and secondly by migration in SDS electrophoresis compared with appropriate standards (13). In this fashion then, on the basis of both exclusion volume and charge density, we will be able to determine with some precision the molecular weight of the chalone (7).

Finally, the carbohydrate content and amino acid content of the purified material will be determined by the use of appropriate chemical and chromatographic techniques as well as automatic amino acid analysis (7).

In summary, all chalones that have been demonstrated to date, namely epidermis, fibroblast, melanocyte, and lymphocyte chalones, are all capable of specifically arresting the mitotic activity of tumor cells of that cell type. Our data, which is probably best defined in the lymphocyte and fibroblast systems, also suggests that while the tumor cell will respond to the chalone, it will not in fact quantitatively be as effective against tumor cells as it is against normal parent cell type. Thus, doses of approximately 3 X larger than normal are required to give equivalent mitotic inhibition for the tumor variety of cells.

Our proposition is that if there is a lung chalone, then one could attempt to use the chalone as an adjunct to chemotherapy on the basis of the following reasons: (a) because of chalone cell specificity, patients receiving amounts of chalone sufficiently large to inhibit the proliferation of lung carcinoma cells in vitro, regardless of their location (such as metastisized cells), would have an inhibition of proliferation of only lung cells for a finite period of time; (b) as the concentration of chalone after a few days was reduced, the tumor cells would escape from chalone control and enter the S phase portion of the mitotic cell cycle. (This proceeds from the fact that most of the chalones we know about inhibit in G_1.); (c) As these tumor cells escape from chalone control and enter S phase in a synchronized fashion, they become enormously vulnerable to those chemotherapeutic agents which function by destroying cells making DNA. (d) further, while tumor cells are entering in a synchronized manner into "S" phase, the normal parent cells of this tumor type will still be under chalone control and hence not entering the "S" phase portion of the mitotic cell cycle. Therefore the normal cells would be largely resistant to chemotherapeutic toxicity and thus the efficiency of chemotherapy would be considerably improved.

It is for this ultimate therapeutic purpose that we seek to search for and purify a lung chalone.

References

1. Houck, et al., Nature New Biol, 240: 210 (1972).
2. Raff and Houck, J. Cell. Physiol., 74: 235 (1969).
3. Houck and Jacob, Proc. Soc. Exp. Biol. Med., 105: 325 (1960).
4. Houck, Ann. N. Y. Acad. Sci., 105: 767 (1963).
5. Houck and Hennings, Fed. Europ. Biochem. Soc Letters, 32: 1 (1973).
6. Zipilivan, et al., Anal. Biochem., 30: 91 (1969).
7. Houck and Cheng, J. Cell. Physiol., 81, 257 (1973).
8. Houck et al., Science, 173:1139 (1971).
9. Houck et al., Clin. Proc. Children's Hospital, 28: 130 (1972).
10. Vesterberg and Swenson, Acta Chem. Scand., 20: 820 (1966).
11. Williams and Reisfeld, Ann. N. Y. Acad. Sci., 121:373 (1964).
12. Piez, Anal. Biochem., 26: 305 (1968).
13. Weber and Osborn, J. Biol. Chem., 244: 4406 (1969).

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Complete cell culture facilities are available as well as a liquid scintillation counter, an isoelectric focusing preparative device, preparative and analytical acrylamide gel electrophoresis device, a cold room, refrigerated centrifuges, refrigerated fraction collectors for column chromatography, automatic amino acid analysis facilities, and the necessary facilities for histology and histochemistry.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Special facilities available (when elsewhere than at the clinical station)

Complete cell culture facilities are available as well as a liquid scintillation counter, an isoelectric focusing preparative device, preparative and analytical acrylamide gel electrophoresis devices, a cold room, refrigerated [illegible] for column chromatography, automatic amino acid analysis system, the necessary facilities

Biographical Sketch

Principal Investigator

Name: John C. Houck

Title: Scientific Director
Research Foundation
Children's Hospital of D. C.

Date & Place of Birth: REDACTED

Education: B.A., 1953
Columbia College, New York

M.S., 1955
University of Western Ontario, Canada

Ph.D., 1956
University of Western Ontario, Canada

Awards: National Research Council (Canada)
Research Fellowships, 1953-1956

New York Academy of Science
A. Cressy Morrison Award, 1962

American Dermatological Association
Research Award, 1965

Societies: REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

REDACTED

Biographical Sketch, John C. Houck, cont'd

Professional Experience:	1969 to Present	Scientific Director Research Foundation Children's Hospital Washington, D. C.
	1969 to Present	Professor of Child Health and Development George Washington University Medical School
	1967-1969	Associate Professor Departments of Pediatrics and Biochemistry George Washington University Medical School
	1967 to Present	Professorial Lecturer Department of Biochemistry Georgetown University School of Medicine
	1960-1967	Associate Professor Department of Biochemistry Georgetown University School of Medicine
	1958-1960	Assistant Professor Department of Biochemistry Georgetown University School of Medicine
	1959 to Present	Director Biochemical Research Laboratory Children's Hospital of D. C.
	1958-1960	Director, Surgical Research Laboratory and Metabolic Laboratory Georgetown University School of Medicine
	1957-1958	Senior Research Fellow Surgical Research Laboratory Georgetown University
	1967 to Present	Editor-in-Chief, INFLO Newsletter of the International Inflammation Club

Biographical Sketch

Research Associate

Name:	Richard F. Cheng
Title:	Research Associate Biochemistry Research Laboratory Children's Hospital of D. C.
Date & Place of Birth:	REDACTED
Education:	D.D.S., 1966 National Taiwan University
	Ph.D., 1972 George Washington University

Professional Experience:

1969 to Present	Research Associate Biochemical Research Laboratory Children's Hospital of D. C.
1968-1969	Research Assistant Department of Biochemistry George Washington University
1967-1968	Dental Resident National Taiwan Univ Hospital
1966-1967	Dental Officer Dental Corps, Chinese Navy

Publications

1. Houck, J. C., Irausquin, H., and Leikin, S., Lymphocyte DNA synthesis inhibition, Science, 173, 1139, 1971.

2. Houck, J. C., Irausquin, H., and Leikin, S., The lymphocyte chalone, Clinical Proceedings of Children's Hospital, 28, 130, 1972.

3. Houck, J. C., Weil, R. L., and Sharma, V. K., Evidence for a fibroblast chalone, Nature New Biology, 240, 210, 1972.

4. Houck, J. C., and Cheng, R. F., Isolation, purification, and chemical characterization of the serum mitogen for diploid human fibroblasts, J. Cell. Physiology, 81, 257, 1973

5. Houck, J. C., and Hennings, H., Chalones: specific, endogenous mitotic inhibitors, FEBS Letters, 32, 1, 1973.

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
John C. Houck	20%	8,000
Richard F. Cheng	100%	16,000
Fringe Benefits @ 8.5% S & W		2,040
Technical		
None		
Sub-Total for A		26,040
B. Consumable supplies (by major categories)		
Cell cultures, media, glassware		3,600
Isotopes and chemicals		1,000
Sub-Total for B		4,600
C. Other expenses (itemize)		
Travel		500
Secretarial		250
Sub-Total for C		750
Running Total of A + B + C		31,390
D. Permanent equipment (itemize)		
Sub-Total for D		
E. Indirect costs (15% of A+B+C)		4,708
Total request		36,098

okay gh

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	27,480	4,600	750		4,924	37,754
Year 3	29,000	4,600	750		5,152	39,502

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Protein chemistry of necrotic wounds	Office of Naval Research N00014-71-C-0203	35,000/yr	6/1/73-5/31/74
Specific mitotic inhibition of leukemic lymphocytes	National Cancer Institute CA12743	60,000/yr	1/1/72-12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Fibroblast chalone	NCI CA 14484	55,000	9/1/73-8/31/76
Immunosuppression of graft rejection by lymphocyte chalone (co-investigator)	NIH	115,000	1/1/74-12/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name John C. Houck, Ph.D.

Signature [signature] Date 7/23/73

Telephone 202 (Area Code) 835-4321 (Number) (Extension)

Checks payable to

earch Foundation of Children's Hospital

Mailing address for checks

2125 - 13th Street, N. W.

Washington, D. C. 20009

Responsible officer of institution

Typed Name Robert V. Morin

Title Executive Director

Signature Robert V. Morin Date 7/23/73

Telephone 202 (Area Code) 835-4011 (Number) (Extension)

10035440 28

1003544029

#875R1 - IOACHIM